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PATENT APPLICATION

VIRAL EXPRESSION VECTORS

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VIRAL EXPRESSION VECTORS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the priority benefit of provisional U.S. Patent Application Serial No. 60/132,697, filed May 4, 1999, pending, which is hereby incorporated herein by reference in its entirety.

TECHNICAL FIELD

This invention is in the field of plant virology. Specifically, the invention relates to the synthesis of nucleic acid sequences encoding an altered viral movement protein, construction of viral vectors expressing such protein, and generation of host plants infected by the viral vectors. The viral vectors permit rapid local and systemic invasion of a host, and allow stable expression of a transgene of interest.

BACKGROUND OF THE INVENTION

In the last fifteen years, considerable progress has been made in expressing foreign genes in plants. Foreign proteins are now routinely produced in many plant species for modification of the plant or for production of proteins for use after extraction. Vectors for the genetic manipulation of plants have been derived from several naturally occurring plant viruses. For the production of specific proteins, transient expression of foreign genes in plants using virus-based vectors has several advantages. Products of plant viruses are among the highest produced proteins in plants. Often a viral gene product is the major protein produced in plant cells during virus replication. Many viruses are able to systemically move from an initial infection site to almost all cells of the plant. Because of these reasons, plant viruses have been developed into efficient transient expression vectors for foreign genes in plants. Viruses of multi-cellular plants are relatively small, probably due to the size

limitation in the pathways that allow viruses to move to adjacent cells in the systemic infection of entire plants. One such plant virus upon which plant expression vectors are based is TMV (tobacco mosaic virus). TMV is the type member of the tobamovirus group. TMV has straight tubular virions of approximately 300 x 18 nm with a 4 nm-diameter hollow canal consisting of approximately 2000 units of a single capsid protein wound helically around a single RNA molecule. Virion particles are 95% protein and 5% RNA by weight. The genome of TMV is composed of a single-stranded RNA of 6395 nucleotides containing five large ORFs. Expression of each gene is regulated independently. The virion RNA serves as the messenger RNA (mRNA) for the 5' genes, encoding the 126 kDa replicase subunit and the overlapping 183 kDa replicase subunit that is produced by read through of an amber stop codon approximately 5% of the time. Expression of the internal genes is controlled by different promoters on the minus-sense RNA that direct synthesis of 3'-coterminal subgenomic mRNAs which are produced during replication. A detailed description of tobamovirus gene expression and life cycle can be found, among other places, in Dawson and Lehto, Advances in Virus Research 38:307-342 (1991).

Thus, it is of scientific and commercial interest to provide new and improved vectors for the genetic manipulation of plants.

SUMMARY OF THE INVENTION

A principal aspect of the present invention is the design of a recombinant viral vector expressing an altered movement protein and altered 126/183 viral proteins to affect stable expression of a transgene in a plant host.

Accordingly, the present invention provides an isolated nucleic acid sequence encoding an altered viral movement protein having the amino acid sequence shown in SEQ ID NOS.: 5 and 6 and altered 126/183 viral proteins. In one aspect, the isolated nucleic acid sequence is essentially identical to the sequence shown in SEQ ID NOS.: 3 and 4, and it contains a Thymine (T) or Uracil (U) residue at position 5212 and Guanine (G) residue at 5303 as shown in Figure 1A. In another aspect, the isolated

nucleic acid sequence is identical to the sequence shown in SEQ ID NOS.: 3 and 4. The alteration of the 30K movement protein and alteration of the 126/183 viral proteins results in an enhanced ability to facilitate stabilization of a transgene contained in a viral vector.

In a separate embodiment, the present invention provides a viral vector comprising the nucleic acid sequence encoding an altered viral movement protein having the amino acid sequence shown in SEQ ID NOS.: 5 and 6 and altered 126/183 viral proteins. In one aspect, the viral vector exhibits an enhanced ability compared to a control viral vector to stabilize a transgene contained in the vector. Preferably, the vector is a tobacco mosaic viral vector. A particularly preferred vector is designated BSG1057 (deposited with American Type Culture Collection having accession number 20398, which was deposited on April 28, 1999).

In a separate aspect within this embodiment, the viral vector comprises a transgene of interest. Preferably the transgene is a non-viral gene encoding a protein selected from the group consisting of a membrane protein, a cytosolic protein, a secreted protein, a nuclear protein, and a chaperon protein.

The present invention also provides a cell transformed with a subject viral vector. The transformed cell may be animal or plant. Preferably, the cell is a plant cell. The present invention further provides a transgenic plant comprising the viral vector. Preferred transgenic plant may, for example, be *Nicotiana benthamiana* or *Nicotiana tabacum*, but others may be just as readily substituted by one of skill in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a comparison of the nucleotide sequences encoding an altered movement protein contained in the vector BSG1057 (SEQ ID NO.: 4) and the wildtype movement protein contained in the vector BSG1037 (SEQ ID NO.: 3). Sequence identities are indicated by *, and mismatches are indicated by -.

Figure 2 depicts a comparison of the amino acid sequences encoding an altered movement protein contained in the vector BSG1057 (SEQ ID NO.: 6) and the wildtype movement protein contained in the vector BSG1037 (SEQ ID NO.: 5). Sequence identities are indicated by *, and mismatches are indicated by -.

Figure 3 is a schematic representation of the restriction sites of the vector BSG1037.

Figure 4 is a schematic representation of the restriction sites of the vector BSG1057.

Figure 5 is the complete sequence of BSG1037 (SEQ ID NO.: 1).

Figure 6 is the complete sequence of BSG1057 (SEQ ID NO.: 2).

Figure 7 is a schematic map of locations of mutations in BSG1057.

Figure 8 shows *N. benthamiana* plants at 20 days postinoculation. There are four columns of five plants. The first column on the left shows plants inoculated with first passage BSG1037. Column 2 is seventh passage BSG1037, Column 3 is first passage BSG1057, Column 4 is seventh passage BSG1057.

MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains. For example, the general teaching for constructing viral plant vectors and using them to systemically infect plants and express heterologous proteins therefrom is disclosed in U.S. Patent Nos. 5,316,931; 5,977,438; 5,889,191; 5,889,190; 5,866,785 and 5,816,653, the entire disclosures of which are hereby incorporated herein by reference.

General Techniques:

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See, *e.g.,* Matthews, PLANT VIROLOGY, 3rd edition (1991); Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

Definitions:

A "plant cell" refers to the structural and physiological unit of plants, consisting of a protoplast and the cell wall.

A "protoplast" is an isolated cell without cell walls, having the potency for regeneration into cell culture, tissue or whole plant.

A "host" encompasses cell, tissue or organism capable of replicating a vector or viral nucleic acid and which is capable of being infected by a virus containing the viral vector or viral nucleic acid. This term is intended to include prokaryotic and eukaryotic cells, organs, tissues, organisms, or *in vitro* extracts thereof, where appropriate. A preferred host cell is a plant cell.

The term "infection" refers to the process of transferring or the ability of a virus to transfer its nucleic acid to a host, wherein the viral nucleic acid is replicated, viral proteins are synthesized, or new viral particles assembled.

The "movement protein" of tobacco mosaic virus is a noncapsid protein required for cell-to-cell movement of the RNA replicons or viruses in plants.

The terms "nucleic acid sequence", "polynucleotide", "nucleotides" and "oligonucleotides" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

As used herein, "expression" refers to the process by which a polynucleotide is transcribed into mRNA and/or the process by which the transcribed mRNA (also referred to as "transcript") is subsequently being translated into peptides, polypeptides, or proteins. The transcripts and the encoded polypeptides are collectedly referred to as gene product. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in an eukaryotic cell.

In the context of polynucleotides, a "linear sequence" or a "sequence" is an order of nucleotides in a polynucleotide in a 5' to 3' direction in which residues that neighbor each other in the sequence are contiguous in the primary structure of the polynucleotide.

A linear sequence of nucleotides is "essentially identical" to another linear sequence, if both sequences are capable of hybridizing to form a duplex with the same complementary polynucleotide. Sequences that hybridize under conditions of greater stringency are more preferred. It is understood that hybridization reactions can accommodate insertions, deletions, and substitutions in the nucleotide sequence. Thus, linear sequences of nucleotides can be essentially identical even if some of the nucleotide residues do not precisely correspond or align. In general, essentially identical sequences of about 40 nucleotides in length will hybridize at about 30^0C in 10 x SSC (0.15 M NaCl, 15 mM citrate buffer); preferably, they will hybridize at about 40^0C in 6 x SSC; more preferably, they will hybridize at about 50^0C in 6 x SSC; even more preferably, they will hybridize at about 60^0C in 6 x SSC, or at about 40^0C in 0.5 x SSC, or at about 30^0C in 6 x SSC containing 50% formamide; still more preferably, they will hybridize at 40^0C or higher in 2 x SSC or lower in the presence of 50% or more formamide. It is understood that the rigor of the test is partly a function of the length of the polynucleotide; hence shorter polynucleotides with the same homology should be tested under lower stringency and longer polynucleotides should be tested under higher stringency, adjusting the conditions accordingly. The relationship between hybridization stringency, degree of sequence identity, and polynucleotide length is known in the art and can be calculated by standard formulae; see, e.g., Meinkoth et al. Sequences that correspond or align more closely to the invention disclosed herein are comparably more preferred. Generally, essentially identical sequences are at least about 50% identical with each other, after alignment of the homologous regions. Preferably, the sequences are at least about 60% identical; more preferably, they are at least about 70% identical; more preferably, they are at least about 80% identical; more preferably, the sequences are at least about 90% identical; even more preferably, they are at least 95% identical; still more preferably, the sequences are 100% identical.

In determining whether polynucleotide sequences are essentially identical, a sequence that preserves the functionality of the polynucleotide with which it is being

compared is particularly preferred. Functionality may be established by different criteria, such as ability to hybridize with a target polynucleotide, and whether the polynucleotide encodes an identical or essentially identical polypeptides. Thus, nucleotide substitutions which cause a non-conservative substitution in the encoded polypeptide are preferred over nucleotide substitutions that create a stop codon; nucleotide substitutions that cause a conservative substitution in the encoded polypeptide are more preferred, and identical nucleotide sequences are even more preferred. Insertions or deletions in the polynucleotide that result in insertions or deletions in the polypeptide are preferred over those that result in the down-stream coding region being rendered out of phase.

The term "hybridize" as applied to a polynucleotide refers to the ability of the polynucleotide to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues in a hybridization reaction. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these.

The terms "cytosolic", "nuclear" and "secreted" as applied to cellular proteins specify the extracellular and/or subcellular location in which the cellular protein is mostly localized. Certain proteins are "chaperons", capable of translocating back and forth between the cytosol and the nucleus of a cell.

A "control" is an alternative subject or sample used in an experiment for comparison purpose. For example, where the purpose of the experiment is to ascertain whether a viral vector carrying an altered movement protein possesses an enhanced ability in systemic invasion of a host plant, it is generally preferable to use a control viral vector (e.g. BSG1037 shown in Figures 1-2) expressing the wildtype altered movement protein (e.g. 1037 sequence shown in Figure 2).

A "cell line" or "cell culture" denotes bacterial, plant, insect or higher eukaryotic cells grown or maintained in vitro. The descendants of a cell may not be

completely identical (either morphologically, genotypically, or phenotypically) to the parent cell.

A "vector" refers to a recombinant plasmid or virus that comprises a polynucleotide to be delivered into a host cell, either *in vitro* or *in vivo*. The polynucleotide to be delivered may comprise a coding sequence of interest in gene therapy. nucleic acid molecule, preferably self-replicating, which transfers an inserted nucleic acid molecule into and/or between host cells. The term includes vectors that function primarily for insertion of a DNA or RNA into a cell, replication of vectors that function primarily for the replication of DNA or RNA, and expression vectors that function for transcription and/or translation of the DNA or RNA. Also included are vectors that provide more than one of the above functions.

A "replicon" refers to a polynucleotide comprising an origin of replication (generally referred to as an ori sequence) which allows for replication of the polynucleotide in an appropriate host cell. Examples of replicons include episomes (such as plasmids), as well as chromosomes (such as the nuclear or mitochondrial chromosomes).

A "transcription unit" is a DNA segment capable of directing transcription of a gene or fragment thereof. Typically, a transcription unit comprises a promoter operably linked to a gene or a DNA fragment that is to be transcribed, and optionally regulatory sequences located either upstream or downstream of the initiation site or the termination site of the transcribed gene or fragment.

Nucleic acids of the present invention

The present invention encompasses a recombinant viral vector expressing an altered movement protein and altered 126/183 viral proteins to effect stable expression of a transgene in a plant host. Distinguished from the previously described movement protein, the altered protein contains two amino acid substitutions (replacing the threonine residue at position 104 with isoleucine, and replacing the lysine residue at position 134 with arginine, see Figure 2). The altered

viral vector exhibits an enhanced ability to facilitate stabilization of a transgene contained in a virus that expresses the altered movement protein.

In one embodiment, the present invention provides an isolated nucleic acid sequence encoding an altered viral movement protein having the amino acid sequence shown in SEQ ID NOS.: 5 and 6 and altered 126/183 viral proteins. In one aspect within this embodiment, the isolated nucleic acid sequence of the movement protein is essentially identical to the sequence shown in SEQ ID NO. 3, and it contains a Thymine (T) or Uracil (U) residue at position 5212 and Guanine (G) residue at 5303 as shown in Figure 1A. As used herein, a linear sequence of nucleotides is "essentially identical" to another linear sequence, if both sequences are capable of hybridizing to form a duplex with the same complementary polynucleotide.

Hybridization can be performed under conditions of different "stringency". Relevant conditions include temperature, ionic strength, time of incubation, the presence of additional solutes in the reaction mixture such as formamide, and the washing procedure. Higher stringency conditions are those conditions, such as higher temperature and lower sodium ion concentration, which require higher minimum complementarity between hybridizing elements for a stable hybridization complex to form. In general, a low stringency hybridization reaction is carried out at about 40 ^{0}C in 6 x SSC or a solution of equivalent ionic strength/temperature. A moderate stringency hybridization is typically performed at about 50 ^{0}C in 6 x SSC, and a high stringency hybridization reaction is generally performed at about 60 ^{0}C in 6 x SSC. The essentially identical nucleic acid sequences embodiment in the invention encompass all sequences encoding modified movement proteins containing conservative or non-conservative substitutions that do not significantly affect the claimed structural characteristics (i.e. retain the substitution of isoleucine for threonine$_{104}$, and arginine for lysine$_{134}$). Modification of polypeptides by altering their corresponding nucleic acid sequences is routine practice in the art. Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tryosine.

Changes in nucleic acid sequence that do not change the encoded amino acid sequence are generally preferred.

The recombinant viral vector embodiment of this invention comprises a nucleic acid sequence encoding the above-described viral movement protein.

In one embodiment, a nucleic acid is introduced into a plant host. Preferably, the nucleic acid may be introduced by way of a viral nucleic acid, using technques known in the art, and preferably the techniques disclosed in U.S. Patent Nos. 5,316,931; 5,977,438; 5,889,191; 5,889,190; 5,866,785 and 5,816,653, the entire disclosures of which are hereby incorporated herein by reference. Such recombinant viral nucleic acids are stable for the maintenance and transcription of such nonnative sequences in the plant host.

BSG1057 (SEQ ID NO.:2) is a mutant version of BSG1037 (SEQ ID NO.:1). The complete sequences of BSG1057 and BSG1037 are shown in FIG. 5 and FIG. 6. BSG1037 has improved insert retention properties.

The difference between these two virus vectors is best demonstrated with the Green Fluorescent Protein (GFP) reporter gene. Both BSG1037 and BSG1057 express GFP which can be visualized under long wave UV light by its green fluorescence. The presence of GFP activity identifies those cells in which the recombinant virus is expressing genes.

Nicotiana benthamiana plants inoculated with BSG1037 and BSG1057 were observed under long wave UV light at approximately 4 to 5 days post inoculation. The GFP spots on the leaves of plants inoculated with the BSG1057 virus were noticeably larger than the GFP spots on the leaves of plants inoculted with the BSG1037 virus, indicating the 1057 virus moves cell to cell faster than BSG1037.

Sequence comparison between BSG1037 and BSG1057

The specific nucleotide changes between 1037 and 1057 are listed in the table below. In those cases where the nucleotide change resulted in an amino acid change, that change is noted (using the single letter code).

nt position	1037 nt	1057 nt	1037AA	1057 AA
1138	A	G	E	G
1268	T	C	No AA changes	
2382	A	G	K	E
3632	G	A	No AA changes	
5213	C	T	T	I
5303	A	G	K	R
5896	C	A	No AA changes	

126/183 refers to the 126/183 viral proteins.
MP refers to the movement protein.

The transgene transcribed by the vector of present invention can be any gene expressed in a biological entity. The selection of transgene is determined largely by the intended purpose of the vector. Preferably the transgene is a non-viral gene selected from the group consisting of a membrane protein, a cytosolic protein, a secreted protein, a nuclear protein, and a chaperon protein.

The vectors embodied in this invention can be obtained using recombinant cloning methods and/or by chemical synthesis. A vast number of recombinant cloning techniques such as PCR, restriction endonuclease digestion and ligation are well known in the art, and need not be described in detail herein. One skilled in the art can also use the sequence data provided herein or that in the public or proprietary databases to obtain a desired vector by any synthetic means available in the art.

Host cell and transgenic organisms of the present invention:

The invention provides host cells transformed with the viral vectors described above. The host cells may be animal or plant, but plant hosts are preferred. The viral vectors containing a transgene of interest can be introduced into a suitable eukaryotic cell by any of a number of appropriate means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is coupled to an infectious agent). The choice of introducing vectors will often depend on features of the host cell.

For plant cells, a variety of techniques derived from these general methods is available in the art. See, for example, U.S. Patent Nos. 5,316,931; 5,977,438; 5,889,191; 5,889,190; 5,866,785 and 5,816,653. The host cells may be in the form of whole plants, isolated cells or protoplasts. Illustrative procedures for introducing vectors into plant cells include Agrobacterium-mediated plant transformation, protoplast transformation, gene transfer into pollen, injection into reproductive organs and injection into immature embryos, leaf abrasion, abrasion in solution, high velocity water spray, and other injury of a host as well as imbibing host seeds with water containing the recombinant viral RNA or recombinant plant virus. As is evident to one skilled in the art, each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant species may not necessarily be the most effective for another plant species.

Agrobacterium tumefaciens-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, bypassing the need for regeneration of an intact plant from a protoplast. The use of Agrobacterium-mediated expression vectors to introduce DNA into plant cells is well known in the art. This technique makes use of a common feature of *Agrobacterium* which colonizes plants by transferring a portion of their DNA (the T-DNA) into a host cell, where it becomes integrated into nuclear DNA. The T-DNA is defined by border sequences which are 25 base pairs long, and any DNA between these border sequences is transferred to the plant cells as well. The insertion of a recombinant plant viral nucleic acid between the T-DNA border sequences results in transfer of the recombinant plant viral nucleic acid to the plant cells, where the recombinant plant viral nucleic acid is replicated, and then spreads systemically through the plant. Agro-infection has been accomplished with potato spindle tuber viroid (PSTV) (Gardner *et al.*, *Plant Mol. Biol.* 6:221 (1986); CaV (Grimsley *et al.*, and Lazarowitz, S., *Nucl. Acids Res.* **16**:229 (1988)) digitaria streak virus (Donson *et al.*, *Virology* **162**:248 (1988)), wheat dwarf virus, and tomato golden mosaic virus (TGMV). Therefore, agro-infection of a susceptible plant could be accomplished with a virion containing a recombinant plant viral nucleic acid based on the

nucleotide sequence of any of the above viruses. Particle bombardment or electrosporation or any other methods known in the art may also be used.

Because not all plants are natural hosts for Agrobacterium, alternative methods such as transformation of protoplasts may be employed to introduce the subject vectors into the host cells. For certain monocots, transformation of the plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments. See, for example, Potrykus et al., *Mol. Gen. Genet.*, **199**:169-177 (1985); Fromm et al., *Nature*, **319**:791 (1986); and Callis et al., *Genes and Development*, **1**:1183 (1987). Applicability of these techniques to different plant species may depend upon the feasibility to regenerate that particular plant species from protoplasts. A variety of methods for the regeneration of cereals from protoplasts are known in the art.

In addition to protoplast transformation, particle bombardment is an alternative and convenient technique for delivering the invention vectors into a plant host cell. Specifically, the plant cells may be bombarded with microparticles coated with a plurality of the subject vectors. Bombardment with DNA-coated microprojectiles has been successfully used to produce stable transformants in both plants and animals (see, for example, Sanford et al. (1993) *Methods in Enzymology*, **217**:483-509). Microparticles suitable for introducing vectors into a plant cell are typically made of metal, preferably tungsten or gold. These microparticles are available for example, from BioRad (*e.g.*, Bio-Rad's PDS-1000/He). Those skilled in the art will know that the particle bombardment protocol can be optimized for any plant by varying parameters such as He pressure, quantity of coated particles, distance between the macrocarrier and the stopping screen and flying distance from the stopping screen to the target.

Vectors can also be introduced into plants by direct DNA transfer into pollen as described by Zhou et al., Methods in Enzymology, **101**:433 (1983). Other techniques for introducing nucleic acids into a plant cell include:

(a) Hand Inoculations. Hand inoculations are performed using a neutral pH, low molarity phosphate buffer, with the addition of celite or carborundum (usually

about 1%). One to four drops of the preparation is put onto the upper surface of a leaf and gently rubbed.

(b) Mechanized Inoculations of Plant Beds. Plant bed inoculations are performed by spraying (gas-propelled) the vector solution into a tractor-driven mower while cutting the leaves. Alternatively, the plant bed is mowed and the vector solution sprayed immediately onto the cut leaves.

(c) High Pressure Spray of Single Leaves. Single plant inoculations can also be performed by spraying the leaves with a narrow, directed spray (50 psi, 6-12 inches from the leaf) containing approximately 1% carborundum in the buffered vector solution.

(d) Vacuum Infiltration. Inoculations may be accomplished by subjecting a host organism to a substantially vacuum pressure environment in order to facilitate infection.

Once introduced into a suitable host cell, expression of the transgene can be determined using any assay known in the art. For example, the presence of transcribed sense or anti-sense strands of the transgene can be detected and/or quantified by conventional hybridization assays (e.g. Northern blot analysis), amplification procedures (e.g. RT-PCR), SAGE (U.S. Patent No. 5,695,937), and array-based technologies (see e.g. U.S. Pat. Nos. 5,405,783, 5,412,087 and 5,445,934).

Expression of the transgene can also be determined by examining the protein product. A variety of techniques are available in the art for protein analysis. They include but are not limited to radioimmunoassays, ELISA (enzyme linked immunoradiometric assays), "sandwich" immunoassays, immunoradiometric assays, in situ immunoassays (using *e.g.*, colloidal gold, enzyme or radioisotope labels), western blot analysis, immunoprecipitation assays, immunoflourescent assays, and PAGE-SDS.

In general, determining the protein level involves (a) providing a biological sample containing polypeptides; and (b) measuring the amount of any immunospecific binding that occurs between an antibody reactive to the trangene

product and a component in the sample, in which the amount of immunospecific binding indicates the level of expressed proteins. Antibodies that specifically recognize and bind to the protein products of the transgene are required for immunoassays. These may be purchased from commercial vendors or generated and screened using methods well known in the art. See Harlow and Lane (1988) *supra.* and Sambrook et al. (1989) *supra.* The sample of test proteins can be prepared by homogenizing the eukaryotic transformants (e.g. plant cells) or their progenies made therefrom, and optionally solubilizing the test protein using detergents, preferably non-reducing detergents such as triton and digitonin. The binding reaction in which the test proteins are allowed to interact with the detecting antibodies may be performed in solution, or on a solid tissue sample, for example, using tissue sections or solid support that has been immobilized with the test proteins. The formation of the complex can be detected by a number of techniques known in the art. For example, the antibodies may be supplied with a label and unreacted antibodies may be removed from the complex; the amount of remaining label thereby indicating the amount of complex formed. Results obtained using any such assay on a sample from a plant transformant or a progeny thereof is compared with those from a non-transformed source as a control.

The eukaryotic host cells of this invention are grown under favorable conditions to effect transcription of the transgene. The host cells may also be employed to generate transgenic organisms such as transgenic plants comprising the recombinant DNA vectors of the present invention. Preferred host cells are those having the propensity to regenerate into tissue or a whole organisms. Examples of these preferred host cells include certain plant cells exemplified herein.

Accordingly, this invention provides transgenic plants carrying the subject vectors. The regeneration of plants from either single plant protoplasts or various explants is well known in the art. See, for example, Methods for Plant Molecular Biology, A. Weissbach and H. Weissbach, eds., Academic Press, Inc., San Diego, Calif. (1988). This regeneration and growth process includes the steps of selection of

transformant cells and shoots, rooting the transformant shoots and growth of the plantlets in soil.

The regeneration of plants containing the subject vector introduced by Agrobacterium tumefaciens from leaf explants can be achieved as described by Fraley et al., *Proc. Natl. Acad. Sci. U.S.A.*, **80**:4803 (1983). In this procedure, transformants are grown in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant species being transformed. This procedure typically produces shoots within two to four weeks and these transformant shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Transformant shoots that rooted in the presence of the selective agent to form plantlets are then transplanted to soil to allow the production of roots. These procedures will vary depending upon the particular plant species employed, as is apparent to one of ordinary skill in the art.

A population of progeny can be produced from the first and second transformants of a plant species by methods well known in the art including cross fertilization and asexual reproduction. Transgenic plants embodied in the present invention are useful for production of desired proteins, and as test systems for analysis of the biological functions of a gene.

EXAMPLES

Tobacco plants inoculated with the viruses BSG1037 or BSG1057 express the reporter gene (GFP) in cells that are infected with either virus. The reporter gene activity (indicative of the presence of virus) is easily observed by illuminating plants with long wave UV light. Viruses that lose expression of the inserted *gfp* gene no longer accumulate the GFP protein and do not exhibit GFP fluorescence under UV illumination.

In order to assess the stability of expression of a foreign gene in the new vector, the *gfp* gene was introduced into the standard vector (giving rise to BSG1037) and the improved vector (giving rise to BSG1057). RNA transcripts of these constructs was generated and used to inoculate *Nicotiana benthamiana* plants. At about 7 days postinoculation, extensive systemic GFP expression was observed. GFP-expressing tissue was harvested, ground in phosphate buffer, the cellular debris removed by low-speed centrifugation, and the resulting "green juice" supernatant solution used to inoculate a new set of *N. benthamiana* plants. Systemic tissue was again harvested at about 7 days and the resulting green juice used to serial passage the virus. The procedure was used to serial passage the viruses a total of 7 times. A comparison was then initiated in which *N. benthamiana* plants were inoculated in parallel with the first passage green juice and the seventh passage green juice for BSG1037 and BSG1057. The first passage virus gave excellent systemic expression of GFP beginning about 4 days post inoculation. The BSG1037 seventh passage virus gave little systemic GFP expression and strong visual TMV mosaic symptoms characteristic of a vector that has lost most or all of the inserted sequence. In contrast, the BSG1057 seventh passage virus still gave excellent systemic GFP expression and the mild visual viral symptoms characteristic of a vector retaining its inserted gene.

At 20 days post inoculation, the plants were cut 2 inches above the soil line and allowed to regrow. Plants were monitored as to the accumulation of GFP protein in the new systemic tissue up to 3 weeks post cutting. Plants containing the BSG 1037 virus (both first and seventh passage) showed very little GFP in regrowth tissues, while showing extensive virus symptoms. This result indicates that the virus population was dominated by viruses that have recombinationally lost the genetic insertion. Plants containing BSG1057 (both first and seventh passage) showed good

systemic invasion in re-growth tissue. This indicates superior genetic stability of the foreign gene insertion, *gfp* gene, in BSG1057 compared with BSG1037.

This increased genetic stability of foreign genes was also seen using two additional gene insertions: interferon gamma from chickens and human alpha galactosidase A. Serial passage experiments of either gene in BSG1037 virus preparations showed variable production of product in plants, while comparable experiments in BSG1057 showed more uniform product accumulation in plants. These experiments indicate that the BSG1057 retains foreign gene insertions through multiple passages to a greater extent.

The invention and the manner and process of making and using it are now described in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, to make and use the same. It is to be understood that the foregoing describes preferred embodiments of the present invention and that modifications may be made therein without departing from the spirit or scope of the present invention as set forth in the claims. To particularly point out and distinctly claim the subject matter regarded as invention, the following claims conclude this specification.